



# An Up-to-Date Overview of the Complexity of Genotype-Phenotype Relationships in Myotonic Channelopathies

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Myotonic disorders are inherited neuromuscular diseases divided into dystrophic myotonias and non-dystrophic myotonias (NDM). The latter is a group of dominant or recessive diseases caused by mutations in genes encoding ion channels that participate in the generation and control of the skeletal muscle action potential. Their altered function causes hyperexcitability of the muscle membrane, thereby triggering myotonia, the main sign in NDM. Mutations in the genes encoding voltage-gated  $\text{Cl}^-$  and  $\text{Na}^+$  channels (respectively, *CLCN1* and *SCN4A*) produce a wide spectrum of phenotypes, which differ in age of onset, affected muscles, severity of myotonia, degree of hypertrophy, and muscle weakness, disease progression, among others. More than 200 *CLCN1* and 65 *SCN4A* mutations have been identified and described, but just about half of them have been functionally characterized, an approach that is likely extremely helpful to contribute to improving the so-far rather poor clinical correlations present in NDM. The observed poor correlations may be due to: (1) the wide spectrum of symptoms and overlapping phenotypes present in both groups ( $\text{Cl}^-$  and  $\text{Na}^+$  myotonic channelopathies) and (2) both genes present high genotypic variability. On the one hand, several mutations cause a unique and reproducible phenotype in most patients. On the other hand, some mutations can have different inheritance pattern and clinical phenotypes in different families. Conversely, different mutations can be translated into very similar phenotypes. For these reasons, the genotype-phenotype relationships in myotonic channelopathies are considered complex. Although the molecular bases for the clinical variability present in myotonic channelopathies remain obscure, several hypotheses have been put forward to explain the variability, which include: (a) differential allelic expression; (b) trans-acting genetic modifiers; (c) epigenetic, hormonal, or environmental factors; and (d) dominance with low penetrance. Improvements in clinical tests, the recognition of the different phenotypes that result from particular mutations and the understanding of how a mutation affects the structure and function of the ion channel, together with genetic screening, is expected to improve clinical correlation in NDMs.

**Keywords:** myotonia, channelopathies, clinical and genetic variability, clinical correlations, functional analyses

## NEUROMUSCULAR DISEASES

Neuromuscular diseases are a clinically, genetically, and biochemically heterogeneous group of more than 80 different entities (<https://www.mda.org/disease/list>), some of which share clinical and dystrophic features (1, 2). The main tissue affected (some diseases are multisystemic) in these diseases is the skeletal muscle, which is the organ in charge of locomotion and other body movements, and contributes to metabolic energy in multicellular organisms. Malfunction of this organ due to structural, physiological, or biochemical changes, often caused by specific genetic mutations, can lead to progressive muscle weakness/wasting with detrimental health consequences (3). Clinical features, such as disease severity, progression, age of onset of symptoms, and prognosis are highly variable (2). Hereditary neuromuscular diseases have been classified into several groups depending on the group of muscles targeted by specific gene mutations. However, clinically different phenotypes have been found in various patients with different mutations in the same gene, and even in separate patients carrying identical mutations (4, 5). The respective genes encode structural proteins, enzymes, ion channels, some of them causing muscular dystrophies (1–3). Muscular dystrophies, which can be inherited as dominant or recessive diseases, include limb girdle muscular dystrophies, congenital muscular dystrophies, dystrophinopathies, facioscapulohumeral muscular dystrophy, Emery–Dreifuss muscular dystrophies, and myotonic dystrophies. This review will focus on a group of disease that have been classified within the group of myotonic conditions, more specifically, the myotonic channelopathies.

## MYOTONIC DISEASES

Electrical properties of the skeletal muscle fiber membrane (in a wide variety of different organisms) are characterized by a high resting membrane permeability for  $\text{Cl}^-$  ions. These  $\text{Cl}^-$  ions are transported by  $\text{Cl}^-$  ion channels belonging to the CLC family, which includes  $\text{Cl}^-$  ion channels and  $\text{Cl}^-/\text{H}^+$  exchangers that are found in all phyla from bacteria to mammals (6–8). The CLC-1 channel is specifically present in skeletal muscle where it accounts for the chloride conductance,  $G_{\text{Cl}}$ , which amounts to ~80% of the resting membrane conductance in resting muscles (9–15). Due to this high conductance and because the equilibrium potential of  $\text{Cl}^-$  is close to the muscle resting membrane potential, CLC-1 conducts membrane currents that inhibit muscle excitability (6). The effective  $\text{Cl}^-$  homeostasis is central in the generation and propagation of the action potential in muscle fibers.

Indeed, it is believed that the prominent role of  $G_{\text{Cl}}$  in action potential repolarization and membrane potential stabilization is related to the large cell size and the peculiar t-tubular system of skeletal muscle. If repolarization were mediated exclusively by  $\text{K}^+$  channels (as in most neurons), the extracellular  $\text{K}^+$  concentration in the restricted t-tubular space would rise significantly, leading to depolarization and eventual inactivation of  $\text{Na}^+$  channels (15). In fact, the “myotonic runs” of repeated action potential firing are partially caused by  $\text{K}^+$  accumulation (15). Importantly, a still open question is whether CLC-1 channels

are actually preferentially located in the t-tubular membrane or in the surface sarcolemma (16–19).

It is well-known that excitation-contraction coupling, triggered by the nerve-impulse induced muscle action potential, is orchestrated by multiple factors (mainly ion channels) that lead to the release of  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum. However, changes in the electrical properties of an excitable muscle, that can occur by both acquired or inherited bases, are well-recognized causes of muscle malfunction in humans (20). Reduction in muscle excitability may be the major cause of muscle weakness leading to fatigue (21), while hyperexcitability may lead to a sustained bursts of discharges that cause involuntary after-contractions, a phenomenon known as myotonia, the classical and leading sign of several hereditary diseases of skeletal muscle (20, 22). Therefore, myotonia is a clinical sign of skeletal muscle that results from an increased excitability of the muscle fiber membrane such that a single nerve stimulus triggers a burst of repetitive action potentials causing a delay in the temporal course of muscle relaxation (23). In patients, this condition can be detected as both electrical and clinical myotonia (24, 25). Electrical myotonia is detected on electromyographic (EMG) tests as repetitive muscle fiber potential discharges, with waxing and waning frequency and amplitude with a firing rate between 20 and 80 Hz, while clinical myotonia is physically demonstrated by slowed muscle relaxation during repetitive hand grip, eye closure, or after tapping various muscles, such as the finger extensors (26).

Symptomatically, patients experience myotonia as stiffness at the beginning of motion, better exemplified during initial attempts at relaxation of hand grip (grip myotonia) and following percussion of the muscle located in the bases of the thumb (percussion myotonia) (27). Myotonia can ameliorate with repeated movements, a phenomenon known as warm-up, but also, it can get worse with activity and become paradoxical (28, 29).

Diseases associated with this sign are collectively termed myotonias and according to other clinical features (progressive muscle wasting and weakness, dystrophic changes), they are classified as: (1) dystrophic myotonias (DM) and (2) non-dystrophic myotonias (NDM). Myotonic dystrophy type 1 (DM1) and type 2 (DM2), caused by the expansion of unstable microsatellites, belong to the first group, and are beyond the scope of this review. A group of five diseases, collectively called myotonic channelopathies, caused by mutations in voltage-gated  $\text{Na}^+$  and  $\text{Cl}^-$  channel genes, belong to the second group (30). This review is focused on the latter group of diseases.

## NON-DYSTROPHIC MYOTONIAS

Dominantly or recessively inherited disorders caused by ion channel dysfunction include myotonia congenita (MC) (Thomsen’s disease and Becker type myotonia), paramyotonia congenita (PC), hyperkalemic periodic paralysis (hyperPP) with myotonia and the sodium channel myotonias (SCM). These diseases show distinctive clinical features that allow separating them from the myotonic dystrophies. They are divided into

two groups: the chloride and the sodium channelopathies. The first group, called myotonia congenita (MC), is subdivided in Thomsen's disease and the Becker generalized myotonia. The other group of three diseases listed above belong to the sodium channelopathies (24, 25, 30–32).

## PHENOTYPE OF MYOTONIC CHANNELOPATHIES

### Chloride Channelopathies

For precise control of muscle contraction by nerve activity, in normal conditions, a single nerve action potential triggers only a single muscle action potential. This is to a large extent guaranteed by the large  $G_{Cl}$  that aids in repolarizing the action potential and, once repolarized, continues to stabilize the membrane potential, preventing thereby the insurgence of a train of action potentials (33). Reduction of the  $Cl^-$  conductance in the skeletal muscle causes myotonia congenita (MC), the most common ion channel disease (34). The first description of this pathology dates back to the late nineteenth century, when Danish physician Asmus Julius Thomsen described it for himself and for some of his family members, with an autosomal dominant inheritance pattern (35). The dominant form of MC was called Thomsen's disease (DMC) after that (36). Almost a century after that first description, German professor Peter Emil Becker described a MC variant with autosomal recessive inheritance pattern, a variant that was called recessive generalized myotonia or Becker myotonia (RMC) (37). MC is electrophysiologically characterized by presenting increased excitability of the muscular fiber, which is due to repetitive action potentials of the muscle membranes; this is reflected in clinical myotonia, muscular stiffness (that is worse after rest) and hypertrophy (38, 39). Myotonia in MC is clinically highly variable, ranging from myotonic discharges only detectable on the EMG test to disabling muscle stiffness at an early age (5). The leg muscles are commonly affected and handgrip myotonia is detected in about  $\frac{3}{4}$  of patients (22). Almost every skeletal muscle in the body might show muscular stiffness, but it is ameliorated by exercise (warm-up phenomenon). The clinical picture depends on whether the disease is inherited as autosomal dominant or autosomal recessive. RMC is more common (in most countries) and severe than DMC (5, 24, 25, 40–42). In DMC, age of onset is usually at birth or very early in infancy. The child might show unusually defined muscles in the extremities, delayed relaxation of the eyelids after forceful closure following sneezing or during crying, and hypertrophy is rare in childhood but common in adulthood (31, 43). Severity varies from mild to moderate, there is no progression of the symptoms, and patients can experience a normal life (43). In RMC, age of onset is usually later than in DMC (25, 43), but it has been also reported that age of onset could be earlier in RMC (44). Myotonia is generalized with moderate to pronounced hypertrophy, where many patients use to have a body-builder appearance due to hypertrophy triggered by the involuntary after-contractions (20, 43). Myotonia is more severe in the recessive variant and usually, typical transient muscular weakness (lasting from seconds to as long as 30 min) is also

observed (10, 45), which may lead to recurrent falls (31, 34). Muscle strength is normal in this variant but the disease slowly progresses in some patients (34, 46).

### Sodium Channelopathies

The upstroke of the action potential is mediated by opening of voltage-gated Nav1.4 sodium channels that generate an inward  $Na^+$  current that renders the cells positive inside (depolarization) (31). Malfunction of these channels cause several human hereditary diseases of the skeletal muscle. Diseases belonging to this group are: paramyotonia congenita (PC), hyperkalemic periodic paralysis (HyperPP) with myotonia and the sodium channel myotonias (SCM). Similar to MC, these diseases are characterized by increased muscle membrane excitability that leads to repetitive action potentials; however, the underlying physiological defect is different compared with the chloride channelopathies (24, 47). Symptoms in sodium channelopathies are episodic and vary from patient to patient and also from time to time on each affected individual (47). Clinical myotonia, affecting hands, face, upper and lower extremities is generalized, going from absent to very severe, depending on the disease, and usually gets worse with exercise; while electrical myotonia may be diffusely present in all muscle and in some cases, may worsen due to cold exposure, which may also induce weakness in some patients, and clinical myotonia. The length of the attack is very variable, lasting from minutes up to 7 days. Muscle hypertrophy is not as common as in the chloride channelopathies, but it is present in variable degrees in some patients. Age of onset of the disease is usually in the first decade and it might progress later in life in some patients (25, 27, 48–50).

PC was first described by Eulenburg (51). Age of onset of first symptoms is at birth or during the first decade of life, affecting mainly the muscles from neck, face and upper limbs. As indicated by its name, PC shows paradoxical myotonia, which gets worse with repeated movements and/or cold exposure. PC show variable weakness, which becomes evident after myotonic stiffness attacks, the major symptom in PC (20, 47). PC patients may experience HyperPP weakness-like phenotype after pronounce cooling or vigorous exercise (20).

In HyperPP, age of onset of the first symptoms (recurrent episodes of weakness as the main symptom) is during childhood. Symptoms are triggered by ingestion of  $K^+$ -rich food, rest after vigorous exercise, and other environmental factors (47, 52–54). The episodes of weakness vary from minutes to several hours, with normal weakness between attacks. Patients affected with HyperPP frequently have myotonia (symptomatically in 12.5% or by EMG in 50% of patients), which can be from mild to moderate, particularly with the onset of a weakness attack. Some patients develop paramyotonia, highlighting the extensive clinical overlapping between these two conditions (20, 47, 53, 55).

SCM is less well-defined with onset of first symptoms from childhood, as in acetazolamide-responsive myotonia, to adolescence, as in myotonia fluctuans (47). Myotonia can be present from mild (in myotonia fluctuans) to severe (in myotonia permanens affecting swallowing and breathing); no periodic paralysis has been observed in SCM patients, while there are

some patients that have shown potassium-aggravated myotonia ( $K^+$ -sensitive myotonia) (20).

## GENETICS AND MUTATION IN MYOTONIC CHANNELOPATHIES

### Chloride Channelopathies

As mentioned above, MC is the most common hereditary ion channel disorder in humans, showing a prevalence between 1:23,000 and 1:50,000 for the recessive form (Becker Myotonia), while the dominant form (Thomsen's disease) is a bit less common in most countries (40, 42, 56). Both conditions are caused by mutations in the chloride voltage-gated channel 1 (*CLCN1*) gene (10, 57–59), which is located in chromosome 7q35 and encodes the voltage-gate chloride channel (ClC-1), belonging to the CLC family of chloride channels (60, 61). Although the pathogenesis of MC is not fully understood, it is well-known that mutations in *CLCN1* produce a reduction of the  $Cl^-$  conductance that leads to membrane hyperexcitability, triggering repetitive action potentials (24, 25, 31). The channel conducts chloride ions over the entire physiological voltage ranges and is the major mediator of chloride conductance in skeletal muscle (13, 14, 31, 62, 63). Two subunits of the channel are required to come together to form the functional channel, and thus, work as double-barreled homodimers (64–66). The *CLCN1* gene has 23 exons, with more than 200 different mutations described in this disease (4, 41, 43, 67, 68) (<http://www.hgmd.cf.ac.uk/ac/index.php>). Mutations are found through the entire gene sequence, being present in the N-terminal, transmembrane, and C-terminal domains of ClC-1. Different types of mutations have been found in the *CLCN1* gene, including nonsense, splice-site, missense, frameshift (insertion/deletions), and deletion/duplication mutations, with exon eight becoming a hot spot for DMC (20, 41, 67, 69–71). The recessive inheritance is conceptually explained by a loss-of-function effect caused by the mutations without significantly impacting on the formation or function of dimeric ClC-1 channels. On the other hand, the dominant inheritance is explained by a dominant-negative effect of mutated subunits on heteromeric mutant/WT channels. Most of the 200 different mutations identified and described behave as recessive, with the majority of the patients being compound heterozygous (carriers of two different recessive mutations). Only about 27 mutations have been associated with DMC, while about other 59 mutations have an unclear inheritance pattern, are sporadic or have been also shown to display a recessive inheritance pattern (<http://www.hgmd.cf.ac.uk/ac/index.php>). Therefore, a clear distinction between dominant and recessive mutations is not always possible (5, 39, 41, 43, 72–74). Thus, far, there is no other clinical phenotype associated with mutations in the *CLCN1* gene.

### Sodium Channelopathies

$Na^+$  channelopathies are not as common as  $Cl^-$  channelopathies, showing a combined prevalence of about 1:100,000 (42). These disorders are caused by mutations in the sodium voltage-gated channel alpha subunit 4 (*SCN4A*) gene, which is located in chromosome 17q23 and encodes the voltage-gated sodium

channel (Nav1.4) of skeletal muscle (75–77). They are a heterogeneous group of autosomal dominant disorders with high penetrance (20, 55). Mutations in *SCN4A* cause disruption of fast inactivation of the channel, which can be incomplete or slowed (78–80), leading to repetitive action potentials (myotonic runs) and consequent intracellular sodium accumulation that depolarizes muscle cells and can lead to inactivation of the  $Na^+$  channels (25, 31, 32, 47). Depending if depolarization is mild or not, myotonia or paralysis might appear, respectively (81). Nav1.4 is a channel formed by a single unit of Nav1.4 protein, which contains four repeated domains (DI–DIV), each one consisting of six transmembrane segments (S1–S6). The loops between S5–S6 segments from the four domains come together to form the ion-conducting pore, acting as a selective filter. Meanwhile, the S4 segment of each domain is in charge of sensing the voltage changes (31, 32, 47). The *SCN4A* gene has 24 exons, with about 83 different mutations described in the gene, but only about 65 of them have been associated with myotonia (40, 82–86) (<http://www.hgmd.cf.ac.uk/ac/index.php>). All *SCN4A* mutations correspond to missense mutations, with the single exception of a deletion/insertion mutation located in the splice site in intron 21 (87, 88). All *SCN4A* myotonia mutations studied produce a gain-of-function effect of Nav1.4, resulting in defects of channel inactivation or enhancement of activation, which explain the dominant inheritance pattern of the diseases (20, 82). Mutations have been located through the entire gene sequence, but depending on the disease, they tend to group differentially. For instance, mutations associated with HyperPP with myotonia are generally located in the inner regions of the transmembrane segments or in the intracellular interlinking loops in repeat domains DII and DIV of the channel, eliciting a persisting inward sodium current, which impairs repolarization and increases membrane excitability (82, 89). In PC, mutations have been found throughout the gene, with exon 24 appearing to be a hot spot (90), but their impairment of fast inactivation is less notorious than the one associated with HyperPP (81). Regarding SCM, although mutations have been found throughout the gene, there are more likely located in the N terminus of the channel, in repeat domain D1, particularly in the inactivation gate (82). Interestingly, in recent papers, the authors describe several *SCN4A* mutations, previously reported in unrelated myotonia-positive families, new or *de novo* mutations, that contribute to apnea during the physiological stress of seizures, severe respiratory failure or associated with paradoxical vocal fold motion (PVFM) (91–93). Neonatal laryngospasm and unusual distribution of myotonia and other NDM signs have also been reported in several NDM patients, who have been shown to carry different *SCN4A* mutations, such as G1306E, I693T, A799S, N1297K, and T1313M (although not all patients that carry these mutations show childhood or neonatal respiratory problems) (78, 94–99). This expands the spectrum of phenotypes associated with mutations in the *SCN4A* gene. In addition to myotonic diseases associated with *SCN4A* mutations, other diseases, that are beyond the scope of this review, also present mutations in this gene, such as: hyperkalemic periodic paralysis without myotonia (HyperPP), hypokalemic periodic paralysis (HypoPP), normokalemic periodic paralysis (NormoPP), and

congenital myasthenic syndrome (CMS) (20, 82). Recently, it has also been suggested that a subset of cases with sudden infant death syndrome (SIDS) might be due to *SCN4A* mutations, as one report (and the only one thus far) has found novel or very rare functionally disruptive *SCN4A* genetic variants associated with SIDS, although the authors indicate that new studies in other populations are required to confirm their finding (100).

## COMPLEX GENOTYPE-PHENOTYPE RELATIONSHIPS IN MYOTONIC CHANNELOPATHIES

In order to provide accurate prognostic information to the patients and families affected with a hereditary disease, it is essential to have appropriated genotype-phenotype relationships. In the case of the non-dystrophic myotonias, this has been extremely difficult mainly due to two factors: (1) the wide spectrum of symptoms and overlapping phenotypes present in both groups ( $\text{Cl}^-$  and  $\text{Na}^+$  myotonic channelopathies) (24, 25, 47), and (2) both genes, *CLCN1* and *SCN4A*, present high genotypic variability, with more than 200 or 65 different mutations already described, respectively (<http://www.hgmd.cf.ac.uk/ac/index.php>) (23, 43). Although many mutations cause a unique and reproducible phenotype in all patients, some *SCN4A* and *CLCN1* mutations cause similar phenotypes. Most worryingly, for several mutations, very different clinical phenotypes have been found in different carriers of the same mutation (23, 25, 47, 101), severely compromising the genotype-phenotype correlation in NDM. Another important limitation for the improvement of genotype-phenotype correlations has been the lack of a sufficient number of individuals carrying each mutation (23), in particular in the case of novel mutations [such as the very recent study that described seven novel *CLCN1* mutations (68)], which makes the situation even more complex, not without mentioning all those cases that show myotonia or a myotonia-like phenotype but in which the mutation has not been found. It is worth mentioning though, that correlation of mutations with the clinical phenotype gives insights into the pathophysiology of human channelopathies, and although some correlation exists between specific mutations and the associated clinical manifestations, this is vague (47).

The poor correlations are most evident in  $\text{Cl}^-$  channelopathies, where the same mutation can be inherited as dominant or recessive with different clinical manifestations, for example, F167L (39, 67, 68, 102, 103), A313T (67, 104), or W433R (44) (see **Table 1**). By recording and analyzing the ion currents of heterologously expressed mutant channels in different *in vitro* expression systems (*Xenopus* oocytes or HEK cells), much progress has been made in understanding how specific mutations affect the function of a particular ion channel, in providing insights onto the mechanism for the inheritance pattern, but also in the role that the voltage-gated ion channels play in excitable tissues (20, 25, 105). These analyses have contributed to improve to some extent, the clinical correlations through a better understanding of the channel dysfunction and its associated clinical picture. Nevertheless,

of the more than 200 or 65 different  $\text{Cl}^-$  and  $\text{Na}^+$  mutations, only about 80 and 30 different mutations, respectively, have been functionally characterized (<http://www.hgmd.cf.ac.uk/ac/index.php>). However, these functional analyses have contributed to understand in a better way the recessive or dominant behavior of different *CLCN1* mutation than the overlapping phenotypes and clinical variability shown by some specific mutation (39, 67, 102, 103). For instance, in general, it has been reported that several recessively inherited *CLCN1* mutations show biophysical defects like reduced open probability, reduced single-channel conductance, or biochemical instability, in a manner that does impinge in a significant manner on the formation or function of heteromeric mutants/WT channels (13, 106, 107). A simple example are early stop codon mutations which do not result in the expression of  $\text{ClC-1}$  subunits (108). In this regard, it has to be remembered that to provoke myotonia,  $G_{\text{Cl}}$  has to be lowered below roughly 30% (109–112). On the other hand, several dominantly inherited *CLCN1* mutations have been shown to exert *in vitro* a dominant negative effect in co-expression with WT (39, 58, 64, 107, 113, 114). In many cases the dominant negative effect is mediated by a shift of the open probability of the “common gate” to more positive voltages (107, 113, 114). These properties provide a rationale to explain the dominant inheritance. However, this does not explain the clinical variability observed in both RMC and DMC. Interestingly, there is a group of 12 *CLCN1* mutations (see **Table 2**) in which the functional *in vitro* analyses have not been able to demonstrate differences with the WT channel, suggesting the presence of additional factors in skeletal muscle fibers, not present in oocytes/HEK cells, that are involved in the disease. Such factors could be related, for example, to subcellular targeting, a still open question for  $\text{ClC-1}$  (18, 19). For these mutations, a powerful experimental approach would be the generation and analysis of knock-in mice carrying the myotonia-related *CLCN1* mutations, since myotonic mice are an established model for chloride channel myotonia (13, 115). Such knock-in mice could provide important information that cannot be obtained in heterologous expression systems. Additionally, patient derived induced pluripotent stem cells (iPSCs), combined with suitable myogenic differentiation (116), or the use of CRISPR/Cas approaches in order to correct (genome editing) specific mutations (followed by evaluating the off-target activities of CRISPR/Cas systems), might provide future avenues of studying the impact or correction of ion-channel mutations. However, in particular for *CLCN1*, these approaches are complicated by the fact that *CLCN1* expression (*in vivo*) requires fully differentiated and innervated muscle fibers (117). Importantly, many of these approaches also apply for the study of sodium channel mutations (see below).

Clinical correlations in sodium channel myotonic disorders are not as complex as the chloride channel myotonic disorders. As with MC, functional analyses in  $\text{Na}^+$  myotonic channelopathies have contributed to the understanding of the pathophysiological mechanism and improvement of clinical correlation, which are more accurate in this case. These analyses have been able to provide that  $\text{Na}^+$  channel mutations have four major effects on Nav1.4 function: (1) enhanced activation;

**TABLE 1** | *CLCN1* mutations behaving as dominant/recessive.

Mutation	Inheritance	Phenotype	Functional effects in heterologous expression systems (references of functional evidence)	References of genetic evidence
c.501C>G, p.F167L	Recessive	Generalized Myotonia (compound heterozygous)	From very small shift of $p_o$ to not different from WT (103, 118)	(39, 102)
	Dominant	Thomsen's like phenotype		(67, 103)
c.689G>A, p.G230E	Recessive	Generalized Myotonia (compound heterozygous)	Dominant negative effect, dramatic change in ion selectivity (58, 114)	(38)
	Dominant	Thomsen's disease		(104, 119)
c.803C>T, p.T268M	Recessive	Generalized Myotonia (compound heterozygous)	Changed $p_o$ of the common gate (120)	(121)
	Dominant	Thomsen's disease		(119)
c.920T>C, p.F307S	Recessive	Generalized Myotonia (compound heterozygous)	Dominant negative effect, shifted the voltage dependence of $p_o$ to positive potentials (74)	(122)
	Dominant	Thomsen's disease		(74)
c.937G>A, p.A313T	Recessive	Generalized Myotonia (compound heterozygous)	Drastically shifted the voltage dependence of $p_o$ to positive potentials (74)	(104)
	Dominant	Thomsen's disease		
c.950G>A, p.R317Q	Recessive	Generalized Myotonia (compound heterozygous)	Shifted gating to positive potentials (113)	(62)
	Dominant	Thomsen's disease		(39)
c.1013G>A, p.R338Q	Recessive	Generalized Myotonia (compound heterozygous)	Shifted the voltage dependence of $p_o$ to positive potentials (118)	(102)
	Dominant	Thomsen's disease		(38)
c.1297T>C, p.W433R	Recessive	Generalized Myotonia (compound heterozygous)	Not determined	(44)
	Dominant	Thomsen's disease		
c.1478C>A, p.A493E	Recessive	Generalized Myotonia (compound heterozygous and homozygous)	Unavailable	(123)
	Dominant	Thomsen's disease		(123)
c.1592C>T, p.A531V	Recessive	Generalized Myotonia (compound heterozygous)	Not determined	(5, 124)
	Dominant	Thomsen's disease		(5)
c.1667T>A, p.I556N	Recessive	Generalized Myotonia (homozygous)	Shifted the voltage dependence of $p_o$ to positive potentials with minimal dominant negative effect (74)	(104)
	Dominant	Thomsen's disease (incomplete dominance)		
c.1936A>G, p.M646V	Recessive	Generalized Myotonia (compound heterozygous)	Unavailable	(123)
	Dominant	Thomsen's disease		(123)
c.2680C>T, p.R894X	Recessive	Generalized Myotonia (compound heterozygous)	Large reduction, but not complete abolition of chloride currents, and weak dominant effects (39)	(39, 121)
	Dominant	Thomsen's disease		(39, 102)

(2) impaired slow inactivation; (3) impaired fast inactivation; and (4) accelerated recovery from fast inactivation. But, as with the *CLCN1* mutations, while these effects explain the dominant behavior of the mutations (dominant gain-of-function effect), they contribute little to the explanation of the clinical variability seen in this group of NDM. Nevertheless, it is well-accepted that: (1) a large fraction of persistent current and an incomplete slow inactivation of the channel may cause a strong long-lasting depolarization, providing the bases for weakness in HyperPP; (2) a slowing of fast inactivation and an incomplete closure of the channel may explain the paradoxical myotonia characteristic of PC; and (3) an increased persistent fraction and/or slowing of fast inactivation might explain the slight depolarization that causes myotonia in affected patients with SCM [reviewed in (47)]. But this does not explain the clinical variation observed in different patients, or even the overlapping symptoms seen with MC. In the case of *SCN4A* mutations, to the best of our knowledge, there have not been reported mutations that, functionally, behave as the WT channel.

Interestingly, there have been few patients carrying a *SCN4A* mutation who have been shown to carry a second mutation in *CLCN1* (see **Table 3**). These patients have shown an exacerbated

or atypical  $\text{Na}^+$  channel disease phenotype, suggesting that both mutation may act synergistically to influence the clinical and neurophysiological phenotype observed in those patients (130). For many years, it was common practice to screen just one among *CLCN1* or *SCN4A* genes, based on the presumptive clinical phenotype. It is thus likely that a significant group of the first (or even some of recent cases) NMDs cases (with atypical/unclear phenotype, unclear inheritance pattern or dual inheritance-dominant or recessive) with molecular diagnosis, carry a second mutation in any of this ion channel genes, and, maybe even in other loci. Based on this and suggested by many authors, the recommendation is that in those NDM cases with atypical phenotypes or inheritance pattern, both *CLCN1* and *SCN4A* genes are screened. However, thanks to new technologies and price reduction, it might now be possible to carry out deep sequencing, such as whole genome sequencing or gene-targeted sequencing (by next generation sequencing-NGS), in these patients. These new approaches not only would allow to properly screen these genes and genotype NDM patients, but also, could contribute to identify gene modifiers that might be involved in modulating their phenotypes. In fact, very recently it was published the first report using whole genome sequencing for screening the *CLCN1* gene in RMC patients (131). These

**TABLE 2** | *CLCN1* mutations behaving similar to CIC-1 WT in *in vitro* expression systems.

Mutation	Phenotype	Functional analysis result	Heterologous expression system	References
c.209C>T, p.S70L	Compatible with RMC	Macroscopic current amplitudes and current slopes comparable to WT	HEK293 cells	(125)
c.244A>G, p.T82A	Compatible with RMC	No effect on chloride currents, very similar to WT	tsA201 cells	(126)
c.313C>T, p.R105C	Compatible with RMC	Chloride currents similar to WT	HEK293 cells, <i>Xenopus</i> oocytes	(103, 106)
c.352T>G, p.W118G	Myotonia positive patients	Currents amplitudes similar to WT	HEK293 cells	(127)
c.449A>G, p.Y150C	Compatible with RMC	Currents indistinguishable from WT	<i>Xenopus</i> oocytes	(107)
c.501C>G, p.F167L	Compatible with RMC or DMC	Currents very similar to WT	HEK293 cells, <i>Xenopus</i> oocytes	(103, 106)
c.782A>G, p.Y261C	Compatible with RMC	Currents indistinguishable from WT	<i>Xenopus</i> oocytes	(107)
c.979G>A, p.V327I	Compatible with RMC	Currents very similar to WT	<i>Xenopus</i> oocytes	(61)
c.1357C>T, p.R453W	Compatible with RMC	No effect on chloride currents, very similar to WT	tsA201 cells	(126)
c.1412C>T, p.S471F	Myotonia positive patients	Electrophysiological parameters similar to WT	<i>Xenopus</i> oocytes	(128)
c.1883T>C, p.L628P	Compatible with RMC	Currents very similar to WT	tsA201 cells	(129)
c.2533G>A, p.G845S	Myotonia positive patients	Currents indistinguishable from WT CIC-1	tsA201 cells	(108)

RMC, recessive myotonia congenita; DMC, dominant myotonia congenita; WT, wild type; NDM, non-dystrophic myotonia.

**TABLE 3** | Simultaneous mutations in *CLCN1* and *SCN4A* in NDM patients showing an atypical phenotype.

Mutations	Phenotype	Heterologous expression system/functional data	References
<i>SCN4A</i> c.3917G>A, p.G1306E <i>CLCN1</i> c.1453A>G, p.M485V <i>SCN4A</i> c.4010G>C, p.R1337P <i>CLCN1</i> c.803C>T, p.T268M <i>SCN4A</i> c.2079T>G, p.I693M <i>CLCN1</i> c.2926C>T, p.Arg976X	Patient 1. Myotonic discharges with type II electrophysiology pattern. PC-like phenotype. Some signs of MC Patient 2. Myotonic discharges with type II electrophysiology pattern. PC-like phenotype. Some signs of MC Patient 3. Myotonic discharges with type III electrophysiology pattern. SCM-like phenotype. Some signs of MC	ND	(132)
<i>SCN4A</i> c.3870C>A, p.F1290L <i>CLCN1</i> c.2848 G>A, p.E950K	Myotonic discharges with type III electrophysiology pattern. SCM-like phenotype with periodic paralysis	HEK293 cells/ <i>SCN4A</i> = enhanced activation- <i>CLCN1</i> = ND	(133)
<i>SCN4A</i> c.3890A>G, p.N1297S <i>CLCN1</i> c.501C>G, p.F167L	Mild NDM phenotype. SCM-like phenotype	HEK293 cells/ <i>SCN4A</i> = impairment of fast and slow inactivation- <i>CLCN1</i> = ND	(134)
<i>SCN4A</i> c.665G>A, p.R222Q <i>CLCN1</i> c.1650G>A p.T550T	Patient with severe myotonia and without fulminant paralytic episodes	HEK293 cells/ <i>SCN4A</i> = enhanced activation- <i>CLCN1</i> = ND	(135)

MC, myotonia congenita; PC, paramyotonia congenita; SCM, sodium channel myotonias; NDM, non-dystrophic myotonia; ND, not determined.

new approaches could be very useful to screen those patients with atypical phenotypes and those in which the effect of the identified mutation does not differ from the wild-type channel (see below).

The molecular bases for the clinical variability present in myotonic channelopathies remain obscure. Yet, a deeper understanding is needed to improve genotype-phenotype correlations. Several hypotheses have been put forward to explain the clinical variability, which include: (a) differential allelic expression; (b) *trans*-acting genetic modifiers; (c) epigenetic, hormonal or environmental factors; and (d) dominance with low penetrance (20, 47, 136–138). These putatively acting (alone or in combination) mechanisms might contribute to explain why the same mutation causes different degrees of channel dysfunction in different patients (74), and therefore elicit a modulation in the NDM phenotype. Importantly, to the best of our knowledge, none of these possibilities has been established experimentally in NDM patients or are under current investigation.

The above considerations suggest that it is not only important to study as many individuals or mutations as possible, but also to try to obtain functional data for all mutations. In addition, deeper insight is expected from bioinformatics and structural approaches combined with information on nearby mutations, aided by the recently obtained 3D structure of the CIC-1 protein (139, 140). The results of these studies, in addition to explaining in a better way the different symptoms associated with particular mutations, would contribute to improving clinical correlations. Improvements in clinical tests, the recognition of the different phenotypes that result from particular mutations and the understanding of how a mutation affects the structure and function of the ion channel, together with genetic screening, is expected to improve clinical correlation in NDMs, which could ultimately be translated into better clinical management and to a better quality of life of affected patients and their families.

Finally, a solid knowledge of functional defects caused by specific mutations might help in the development of selective drugs targeted at a correction of such effects. For example,

gating modifier drugs could be developed to invert the shift of the voltage-dependence of some dominant *CLCN1* mutations. Several small molecule compounds are indeed known to interfere with the open probability of ClC-1 channels, suggesting the principal feasibility of such an approach (141, 142). In particular, derivatives of clofibric acid have been shown to shift the voltage-dependence of ClC-1 and the related ClC-0 to more positive voltages (141–143). The development of novel molecules that specifically bind to and stabilize the open state of the channel could be aided by the recent determination of the structure of ClC-1 (139, 140). Another possible line of intervention regards patients with mutations that lead to protein folding defects. Such defects might be treated with small molecules that act as “correctors.” Such an approach is already in clinical use in other diseases, such as cystic fibrosis (144). Since correctors are assumed to stabilize the folded state of the channel, it might be tempting to start the search of correctors using ClC-1 inhibitors, like 9-anthrazene carboxylic acid (9AC) (143). However, all known ClC-1 inhibitors are of rather low affinity and quite unspecific (8). Therefore, such correctors are likely only to be discovered using high-throughput screening, as done for the

most common CFTR mutation causing cystic fibrosis (118). Indeed, generic correctors, such as 4-phenylbutyrate, miglustat, and sildenafil have proven unsuccessful for cystic fibrosis (118).

For preparing this review, we (both authors) have used our personal historical literature database of publications regarding *CLCN1* and *SCN4A* related myotonia, as well as PubMed searches using the keywords “myotonia AND (CLCN1/CLC-1, SCN4A/Nav1.4, apnea, laryngospasm).”

## AUTHOR CONTRIBUTIONS

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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